OPTIMIZATION OF FINGER LAKES RIESLING'S FERMENTATION KINETICS BY VARIATION OF NUTRIENT TYPE, TEMPERATURE AND YEAST STRAIN

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By

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ABSTRACT

Yeast Assimilable Nitrogen, abbreviated as YAN, plays an essential role in the metabolic processes of yeast during grape juice fermentation. Previous work suggested that for Riesling fermentations in the Finger Lakes (FLX) of New York, a concentration of 150 mg N/L was sufficient for the fermentation to reach dryness (Tahim, 2016).

To further investigate other variations and environmental conditions that might affect the fermentation kinetics of cool-climate Riesling, a series of fermentations was carried out in a chemically defined grape juice media, formulated to reflect the chemical composition of a typical Finger Lakes Riesling. The YAN concentration was adjusted to 150mg N/L with two different nutrient sources and a control treatment maintained with no nutrient added; all three nutrient conditions were fermented at three temperatures with two yeast strains commonly used in the FLX wine region. Nitrogen sources include diammonium phosphate (DAP), an inorganic ammonium salt commonly used in the wine industry, and a liquid mixture of amino acids (an organic nutrient source) formulated according to the amino acid profile of a typical Finger Lake Riesling. Temperature was set at 23°C (high), 18°C (moderate), and 12°C (low), and the commercial *Saccharomyces cerevisiae* yeast strains EC1118 and WC15 were used. Together 18 treatments were implemented, with each treatment carried out in duplicates, resulting in a total of 36 individual fermentations. Fermentation kinetics were determined by analyzing the rate of YAN depletion over the course of fermentation, and measuring grape juice chemistry parameters including organic sugars and acids. The aim of the study is to better understand how, and to what degree, variations in temperature, yeast strain and nutrient source affect fermentation kinetics and the chemical composition of the final wine.

Of the three factors, yeast strain had the greatest impact on the pattern of sugar depletion over the course of fermentation, with EC1118 showing a faster biomass accumulation and faster sugar consumption rate during onset of fermentation. Temperature had the greatest impact on the length of fermentation, with 23°C having the shortest fermentation length and 12°C the longest. The rate and pattern of YAN consumption was universal, regardless of nutrient type and yeast strain, though 12°C treatments showed a slightly slower depletion rate. Nutrient type, temperature and yeast strain were found to have an interactive effect on multiple wine chemistry parameters, especially on the final concentration of acetic acid, malic acid, residual sugar and ethanol.

These data provide wine producers in cool-climate regions a better understanding of the interaction of various factors during Riesling wine production.

BIOGRAPHICAL SKETCH

Ray Ruiming Chen was born in Zhuhai, China and raised in France and New Zealand. She developed a deep interest in winegrowing during her childhood stay in France, and graduated in 2015 from Lincoln University, in Christchurch, New Zealand with a Bachelor of Viticulture and Oenology double major in Marketing. Upon graduation, she continued to work in the wine industry, in several New Zealand and Californian wineries, Wines and Spirits Education Trust in London, as well as a fine wine retail company in Shanghai, China. In 2017, Ray began her Master's study in Food Science and Technology, specializing in Enology under the supervision of Dr. Anna Katharine Mansfield. The following research project directly pertains to her scientific enthusiasm for solving problems and developing commercially practicable methodologies in the pursuit of higher-quality winemaking.

ACKONWLEDGEMENT

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INTRODUCTION

Yeast assimilable nitrogen (YAN), consisting of primary amino nitrogen (PAN) from amino acids and ammonium ions (AMM), is the nitrogenous fraction available for metabolism of *Saccharomyces cerevisiae*. It is essential for accumulation of yeast biomass during onset of fermentation and maintains fermentation activity; subsequently, YAN can potentially affect the resulting wine's flavor profile and chemical composition. YAN level in white varieties such as Riesling and Traminette are generally low on the Finger Lakes region of NY, with an average lower than 100 mg N/L annually. The majority of the amino acids in grapes are in the form of proline, a secondary amino acid that can not be metabolized by *Saccharomyces cerevisiae* because it lacks certain extracellular enzymes for the purpose of degradation. Low nutrient levels in white must is also linked to distribution, as around 70% of the solutes and 30% of the total amino acids are distributed within the seeds and skins (Stinesa et al., 2000). For white wines, juice is pressed off the skins and seeds before fermentation, and their YAN content is therefore no longer available to the yeast. Hence, reliance on exogenous nitrogen supplementation is common for Riesling production in the region. Exogenous nitrogen addition facilitates fermentation by either raising the fermentation rate per yeast cell or enhancing the number of cells per population (biomass) (Gutiérrez et al., 2012).

The minimal requirement of YAN is 140 mg N/L; below this threshold, several fermentation issues might arise (Bell & Henschke, 2005). Stuck or sluggish fermentation is one of the most common consequences; the lack of sufficient nitrogen sources supporting yeast metabolism causes fermentation to arrest prematurely, and the rate of sugar depletion declines rapidly, leaving high concentration of residual sugar in the wine ((Maisonnave et al., 2013). Another common consequence of nutrient starvation is the formation of hydrogen sulfite, a volatile compound commonly associated with the smell of rotten egg. A stressful fermentation environment will lead to accumulation of

sulfur-containing amino acids cysteine and methionine by several permeases, and nitrogen deficiency triggers *Saccharomyces cerevisiae* yeast to alter its metabolic pathway to the sulfate assimilation pathway of which cysteine and methionine are degraded to hydrogen sulfite, producing an unpleasant aroma with a low sensory threshold (Kinzurik et al., 2016) (Moreno-Arribas & Polo, 2008).

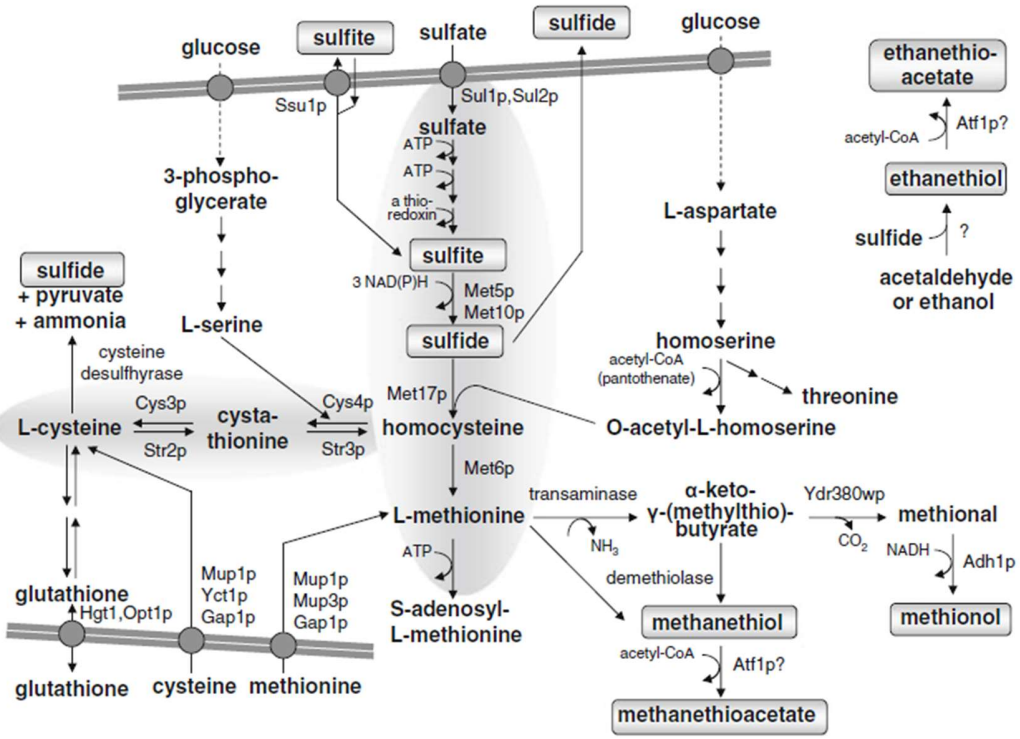

Figure 1. Sulfate assimilation pathway in *Saccharomyces cerevisiae* yeast (Moreno-Arribas & Polo, 2008).

According to several industry protocols, the standard YAN target for a smooth fermentation being able to ferment to dryness is a total of 250 mg N/L. However, this threshold is proposed by wine researchers in California, where they experience very different growing conditions compared to the east coast.

The Finger Lakes wine region is a typical cool climate viticulture area with continental climate influence. The majority of the Finger Lakes vineyards lie between 42°N to 43°N

latitude and benefit from a location adjacent to water body that buffers the cold temperature of the winters (Lergp.org, 2018), however, the average number of Growing Degree Days (GDD) and average season temperature are still considerably lower than California. As of vintage 2016, the accumulated GDD in the Finger Lakes region was around 2800 GDDs, whereas Napa, California had over 3200 GDDs (Hems and Madill, 2016) (Newa.cornell.edu, 2018) (Wine business, 2018). Consequently, Finger Lakes producers harvest grapes at a lower Brix compared to Californian producers, and therefore require a smaller nutrient addition.

Previous research suggests that 150 mg N/L was sufficient for complete fermentation of cool climate Riesling (Tahim, 2016). While the level of nitrogen available for yeast has been proven to both qualitatively and quantitatively alter the fermentation kinetics, wine chemical composition, flavor, and aroma profile (Querol & Fleet, 2006), when modulating a certain wine parameter in commercial winemaking settings, many other factors should also be taken into account. As the interactive effect of these factors have not been well studied for cool climate Riesling, this research focuses on three: nutrient type, fermentation temperature and yeast strains.

Sources of YAN can be either inorganic (in the form of ammonium ions) or organic (in the form of primary amino nitrogen from amino acids); inorganic nitrogen is the most common type of exogenous supplementation added to grape must prior or during fermentation, typically in the form of Diammonium Phosphate (DAP). Organic nitrogen can either originate from the grape, in which case, asparagine, glutamine and glutamate are preferentially metabolized due to their ability to suppress the expression of genes regulating continuous nitrogen uptake and the activities of catabolic enzymes. Alternatively, organic nitrogen can be retrieved from autolyzed yeast and added as an external nitrogen source in commercial products such as Fermaid O and Fermaid K (Scott Laboratories). It is reported that ammonium is the preferred nitrogen source for biomass formation during onset of fermentation, and primary amino nitrogen is

preferred later during stationary phase for yeast cell maintenance (Beltran et al., 2005). When ammonium is added in a high dosage, it can trigger nitrogen catabolite repression (NCR), in which the uptake of amino acids is obstructed (Jiranek & Henschke, 1995) (Bell & Henschke, 2005). Typically, the transcription of genes responsible for the expression of those least preferred amino acids are repressed, consequently inactivating and degrading the corresponding amino acids (Beltran, Rozès & Guillamón, 2007). This can potentially influence the wine aroma profiles since amino acids also act as precursors of many volatile esters. On the other hand, organic nitrogen has been found to increase the production of higher alcohols and mid-chain fatty acids and their corresponding acetate and ethyl esters, which contribute to the fruity and flavor aromas in wines (Beltran et al., 2005). It is also reported that other non-volatile compounds such as glycerol, carboxylic acids, succinic acid and α-ketoglutaric acid vary in concentration depending on nitrogen source and concentration (Vilanova et al., 2007). Fermentation temperature serves as another essential factor which can influence both the rate and pattern of nutrient update, as well as final wine chemistry and flavor profile. Research suggests that nitrogen uptake and yeast biomass both happen at a faster rate during high temperature fermentation, and the acceleration in consumption rate is more prominent when ammonium is the only nitrogen source (Vilanova et al., 2007) (Vilanova et al., 2012).

It has been reported that most white and rosé wines produced at a lower fermentation temperature (10 to 15 °C) developed aroma and flavor profile with higher intensity (Waterhouse, Sacks & Jeffery, 2016). Higher temperatures will speed fermentation kinetics and shorten fermentation length, a cool temperature fermentation seems to yield more aromatic wines. This could be explained in several ways. A low fermentation temperature alters the gas-liquid phase ratio and volatility of the compounds, as well as subsequent carbon dioxide entrainment, therefore reducing losses of those volatile compounds responsible for the fruity and floral aromas in wines - mostly acetate and

ethyl esters, monoterpenes and norisoprenoids (Waterhouse, Sacks & Jeffery, 2016). A low temperature fermentation reduces yeast membrane fluidity and induces structural changes in membrane permeases, the enzymes that regulate the uptake of nitrogen. Subsequently, this will trigger the synthesis of polyunsaturated fatty acids to increase lipid saturation; under an anaerobic winemaking environment, this synthesis is interrupted, releasing mid-chain fatty acids which then react with ethanol in the fermenting juice to produce ethyl esters (Beltran, Rozès & Guillamón, 2007). Since low temperatures slow down kinetics, yeast experience a longer growth phase and more time to produce more volatile compounds. Hydrolysis of acetate and ethyl esters also happens at a faster rate in a high temperature environment. During this process, esters are hydrolyzed back to their corresponding precursors and therefore lose aromatic properties. With an increase in temperature of 10 °C, the rate of hydrolysis will be doubled (Waterhouse, Sacks & Jeffery, 2016).

Multiple studies have shown that lipid metabolism responses vary among *S. cerevisiae* yeast strains. Under stressful fermentation environment and nutrient deficiencies, alteration in plasma membrane composition, mainly modification in lipid structure, occurs as an adaptative response by yeast, and this response defines the viability of a specific yeast strain (Beltran et al., 2008) (reference15). For this study, two commercial yeast strains were chosen due to their popularity among Finger Lakes Riesling producers. EC1118 is originally a champagne yeast isolated from Epernay, France, used primarily for secondary in-bottle fermentation due to its high alcohol tolerance, robust viability and ability to ferment well at low temperatures (Scotts Laboratories, 2018a). W15 is commonly used for the production of Alsatian or aromatic white varieties, such as Riesling, Pinot Gris, Gewürztraminer, as well as fruit wines and French hybrids; it is characterized by its low heat generation during fermentation, which minimizes potential temperature spikes and, consequently, hydrogen sulfite formation (Scotts Laboratories, 2018b).

While the impact of these individual factors, nutrient source, yeast strain and fermentation temperature are well studied among wine researchers, these factors do not independent alter yeast metabolism and operations, and further work is required to better identify the interactive effects of nutrient source, fermentation temperature and yeast strain, and their implications on a given wine parameter quantitatively and qualitatively. This study focused on the optimizing the fermentation parameters of characteristically YAN deficient Finger Lakes Riesling by variation of these factors; in order to maintain a constant grape juice chemical composition for the study of yeast metabolism, synthetic grape juice media was used.

MATERIALS AND METHODS

Experimental Design

The experimental design incorporated 3 different factors: temperature, yeast strain and nutrient source. There were three levels of temperature, 12, 18 and 23 °C. Two types of *S. cerevisiae* yeast strains were used, W15 and EC1118. Nutrient treatments consisted of a control with no nutrient added, diammonium phosphate (DAP), and an amino acid mix. In total there were 18 different combination of treatments (with controls included). Duplicates were carried out for each treatment, resulting in a total of 36 individual fermentations.

Reagents

Synthetic grape juice media (SGJM) was produced according to a formula modified from Wang et al. (2003). The initial sugar concentration of the SGJM was adjusted to 190 g/L. The amino acid proportion in the formula was adjusted according to formula reported by Vilanova et al. (2007), and the SGJM contained an initial YAN of 40ppm. Chemical reagents used for HPLC 1100 analysis included 6% Acetonitrile and 0.045N sulfuric acid (Fisher Scientifics, Pittsburg, PA).

Nitrogen Sources

Two nitrogen sources were used for treatments: diammonium phosphate (DAP) and a mix of amino acids (organic nutrient). Diammonium phosphate was purchased from Fisher Scientific (Waltham, MA). A food-grade amino acid liquid mixture (Sigma-Aldrich, Saint Louis, MO) was formulated according to the Spayd and Andersen-Bagge paper (1996), which reported the average proportion of amino acids in Riesling grape juice.

Fermentations

SGJM was allocated into 250 mL volumetric flasks (Kimax Kimble, Vineland, NJ) and inoculated as per manufacturer instructions. W15 (Scott Laboratories, Petaluma, USA)

and EC1118 (Scott Laboratories, Petaluma, USA) were added according to the standard industry addition rate of 25g/hL; Goferm (Scott Laboratories, Petaluma, USA) was added as a yeast rehydration nutrient at a rate of 0.3g/L. Fermentation flasks were placed in a temperature-controlled Fisher Scientific reciprocal shaking bath for the 18 and 23 °C treatment (Fisher Scientific, Pittsburg, PA) or a Brunswick Scientific C24KC refrigerated incubator shaker for the 12°C treatment (Enfield, Connecticut).

Nutrient addition was performed as per manufacturer instructions, with the first half of the addition at 24 hours post-inoculation to estimate the onset of fermentation or end of lag-phase, and the second at 1/3 sugar depletion. Total YAN level was set at 150 ppm; 40ppm was contributed by the SGJM and 10ppm YAN by GoFerm, making the actual rate of supplementation 100ppm for both nutrient sources. Brix tracking was carried out daily by Anton Paar DMA35 density meter (Ashland, VA) until 1/3 sugar depletion, then every two days until the it reached a Brix of -1, at which point Bayer Clinitest Reagent tablets (Whippany, NJ) were used to determine residual sugar. The fermentation was considered complete if Clinitest returned a negative result (0-0.25%). Any stuck or sluggish fermentation was terminated after the residual sugar concentration remained constant for more than three days, which was also assessed by Clinitest tablets, with sample dilution performed if necessary.

Sampling

Two 2mL samples were taken daily, prior to 1/3rd sugar depletion, using an Alphapette Single Channel 1000 uL Pipettor (MIDSCI, St. Louis) and stored in a -4 Celsius freezer for future analysis. One of each sample was stored in a 2.0 ML MCT certified flat cap, GRD 500/UN Tube (LCP), and the second in a 2.0mL graduated, free standing microcentrifuge tube (Fisher Scientific, Pittsburg, PA). Starting at 1/3 sugar depletion, samples were taken every two to three days and stored in -4 Celsius freezer for future analysis. Upon completion or termination of fermentation, 35mL of the final sample was

taken for analysis of residual sugar (g/L), pH, titratable acidity (g/L) and ethanol content (%). Prior to analysis, samples were thawed and brought up to room temperature by heating the samples in a water bath at 60°C for thirty minutes. Samples were then cooled to room temperature and homogenized by an IKA Vortex3 homogenizer (Wilmington, NC, USA) for further processing.

Instrumental Analyses

Due to the high volume of data, only samples on selected days were analyzed. Primary amino acid (PAN) and Ammonia (AMM) were analyzed using the Randox RX Monaco, model RS-232, an automatic spectrometer (Randox Laboratories, Jefferson County, West Virginia). For PAN, samples were analyzed using a Randox NOPA reagent assay (Randox Laboratories, Jefferson County, West Virginia); the detection range was 10 to 500 mg N/L, and therefore dilution for samples was not required. For AMM, samples were tested by Randox Ammonia reagent assay (Randox Laboratories, Jefferson County, West Virginia); because the ammonia assay had a sensitivity of 0.4 mg N/L, and the maximal detection threshold was 15 mg N/L, a 10 times dilution was performed as necessary. Standards were run every 30 samples in order to ensure the accuracy.

Organic acids (acetic acid, citric acid, tartaric acid, malic acid and lactic) and residual sugars (glucose and fructose) were analyzed using a high-performance liquid chromatography (HPLC) on an Agilent 1100 module complemented by a mass spectrometry (MS) detector.

Samples for HPLC analysis were filtered through a Celltreat syringe filter PES (polyethersulfone) 0.22 μm membrane connected with a BD 1mL tuberculin slip tip syringe (Pepperell, MA, USA) before analysis. A 10-fold dilution was performed for samples which were taken during the early stage of the fermentation due to the high concentration of sugar presented in the juice. Mobile phase solution was used for both sugar and acid analysis, containing 6% acetonitrile, 0.045 N H2SO4, and type 1 water.

Prior to each run, the column was equilibrated with mobile phase running at the rate of 0.5mL/min for an hour; column, guard cartridge and mobile phase were equilibrated to 45°C. Compounds were identified by comparing retention times and mass spectra with chemical standards. For this experiment, compounds of specific interest include: glucose, fructose, acetic acid, citric acid, tartaric acid, malic acid and lactic acid.

Statistical analysis

Rate of sugar depletion and YAN consumption was represented by exponential decay curves based on glucose and fructose concentrations from HPLC analysis, and PAN and Ammonia AMM concentrations from RX Monaco analysis, respectively. For other wine chemistry parameters (organic acids, pH, TA and ethanol content), they were analyzed with a linear model which predicted means based on a factorial design of three nutrient sources (amino acid mix, DAP and control) by three temperatures (12, 18 and 23 °C) by two strains (EC1118 and W15), resulting in a 3x3x2 ANOVA. Post-hoc analyses, pair-wise analysis, Tukey test and estimated predicted means were calculated with the R package emmeans and emmip.

RESULTS

Initial Model Juice Chemistry

The initial chemistry for SGJM was pH 3.27, titratable acidity 4.8 g/L (as tartaric acid equivalent TAE), citric acid 0.134 g/L, tartaric acid 2.51 g/L, malic acid 2.27 g/L, lactic acid 0 g/L, acetic acid 0 g/L, residual sugar 190 g/L, and YAN 40 mg N/L.

Effects of Nutrient Source, Temperature and Yeast Strain on Fermentation Kinetics

A three-way interaction between yeast strain, nutrient source and temperature affected the fermentation rate and pattern of sugar depletion. With all nutrient sources and temperatures, EC1118 fermented faster than W15. For 23°C and 12°C treatments, there were no significant difference between treatments inoculated with the same yeast strains but supplemented with different nutrient sources (DAP and amino acid mix AA). For 12°C treatments, EC1118 treatments supplemented with DAP took 25 days to ferment to dryness, and EC1118 treatments supplemented with AA took 31 days. There were no significant difference in fermentation length between DAP and AA treatments for 12°C EC1118 ($p= 0.4055$). Both the DAP and AA treatments of W15 at 12°C took 43 days to complete fermentation. Both controls took much longer, and neither fermented to dryness; 12°C EC1118 and W15 Controls ceased active fermentation on day 55, which was significantly longer than N addition treatments at the same temperature.

For 18°C treatments, W15 AA treatment had a significantly longer fermentation, and the rate of sugar depletion was much slower. This is likely an outliner, with the slow sugar consumption possibly due to an unsuccessful inoculation and yeast was struggling to build up biomass. W15 DAP, EC1118 AA and EC1118 DAP all had very similar sugar consumption patterns and all fermented to dryness between days 15-20. W15 Control was not able to ferment to dryness and ceased fermentation activity on day 82.

For 23°C treatments, despite the fact that EC1118 was a faster fermenter than W15, all treatments with exogenous nitrogen supplementation fermentation around day 20. Fermentation rate and length varied greatly between replications of the control fermentations for both yeast strains, possibly because replicates were performed at different times. Despite the variation in fermentation rate, all controls fermented to dryness at 23°C.

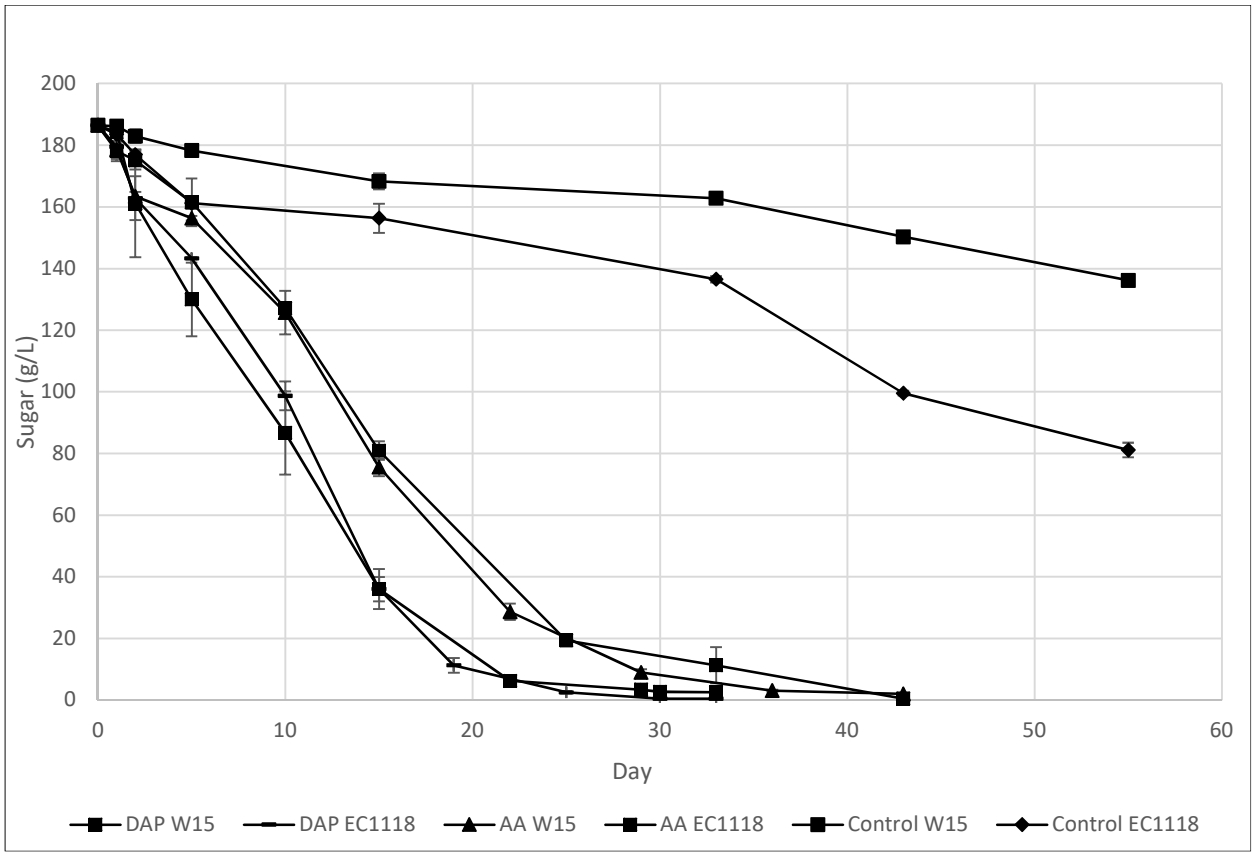

Figure 2. Sugar consumption of SJGM[1] fermentations supplemented with nutrient source DAP, AA and Control, fermented with yeast strains EC1118 and W15, at 12°C. Error bars represent standard error between the two sample replicates.
[1]Synthetic grape juice media.
AA= amino acid mix. DAP = Diammonium phosphate.

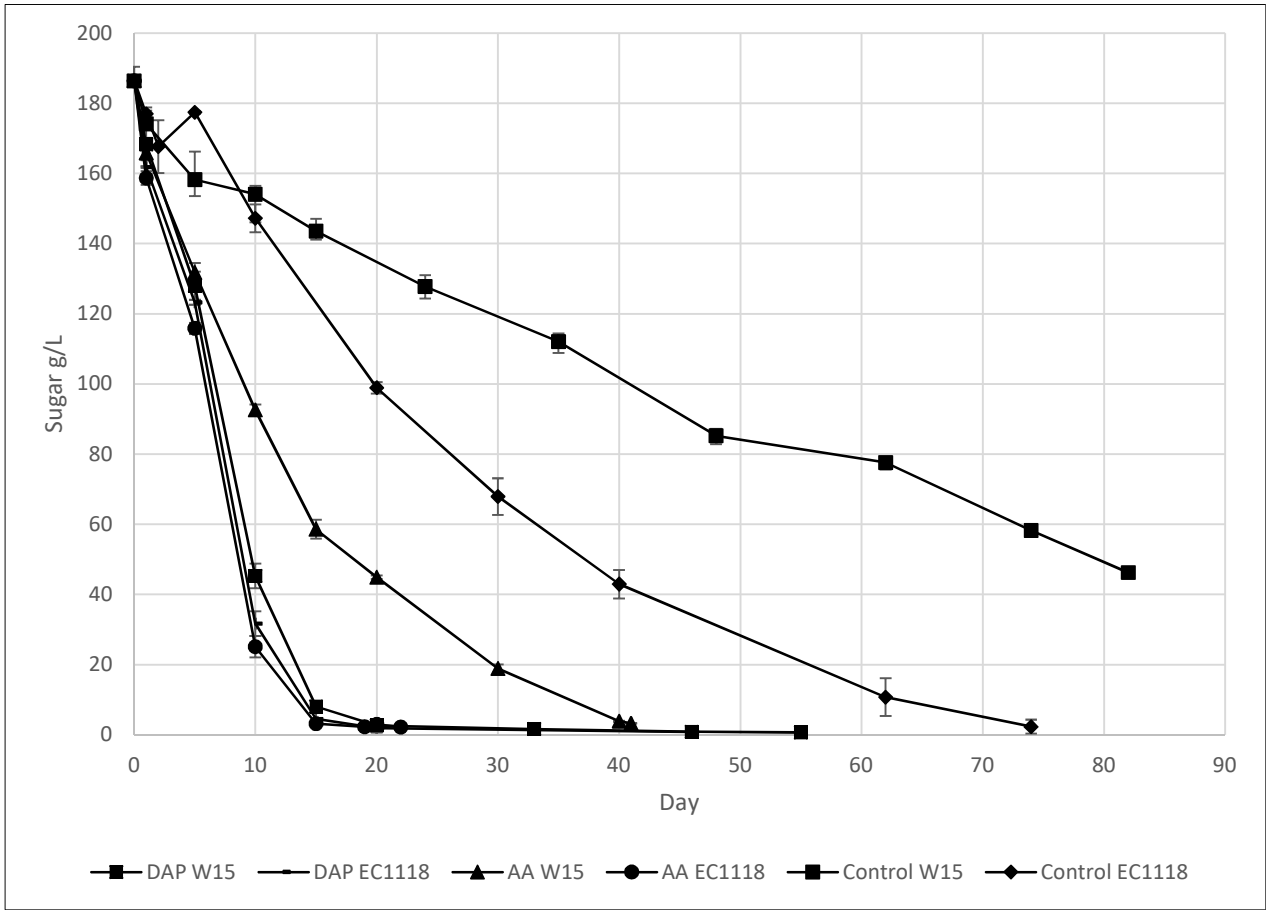

Figure 3. Sugar consumption of SJGM[1] fermentations supplemented with nutrient source DAP, AA and Control, fermented with yeast strains EC1118 and W15, at 18°C. Error bars represent standard error between the two sample replicates.
[1]Synthetic grape juice media.
AA= amino acid mix. DAP = Diammonium phosphate.

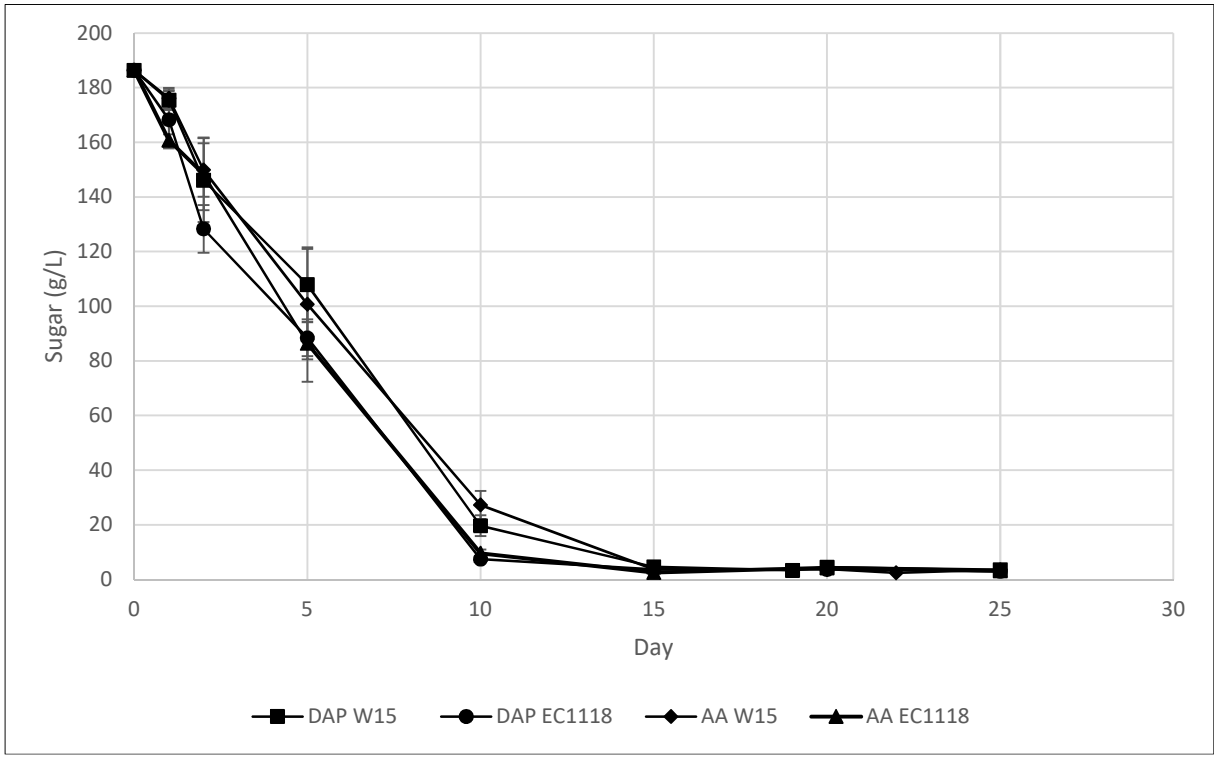

Figure 4. Sugar consumption of SJGM[1] fermentations supplemented with nutrient source DAP and AA, fermented with yeast strains EC1118 and W15, at 12°C. Error bars represent standard error between the two sample replicates.
[1]Synthetic grape juice media.
AA= amino acid mix. DAP = Diammonium phosphate.

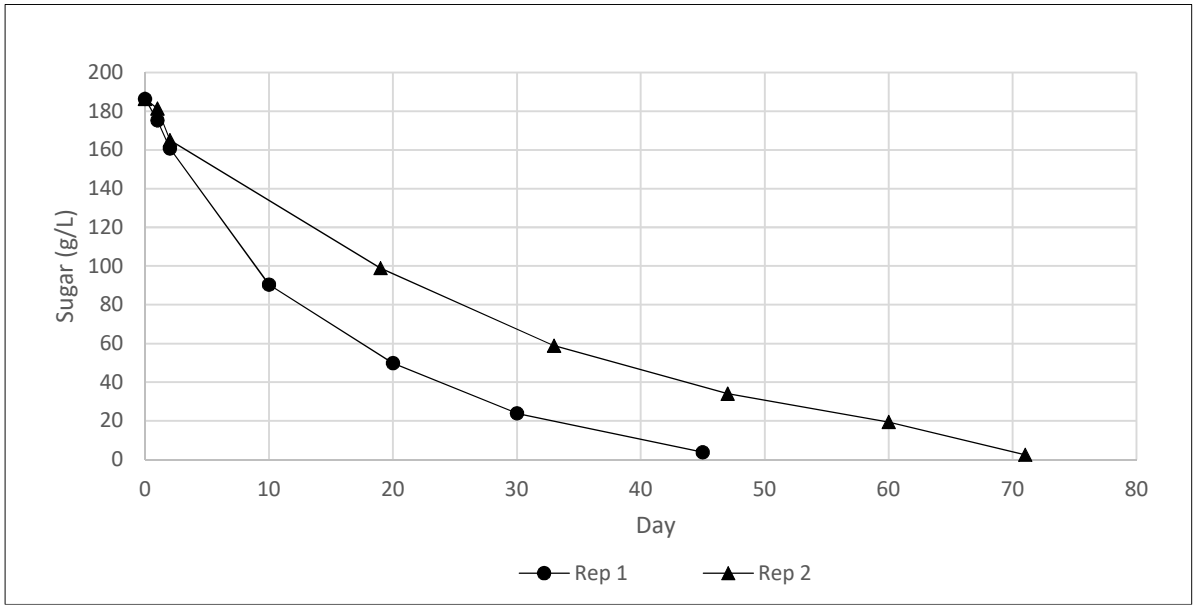

Figure 5. Sugar consumption of SJGM[1] fermentation replicates with no exogenous nitrogen added (control), fermented with strain EC1118, at 23°C.
[1]Synthetic grape juice media.
Rep 1 = replicate 1, rep 2 = replicate 2.

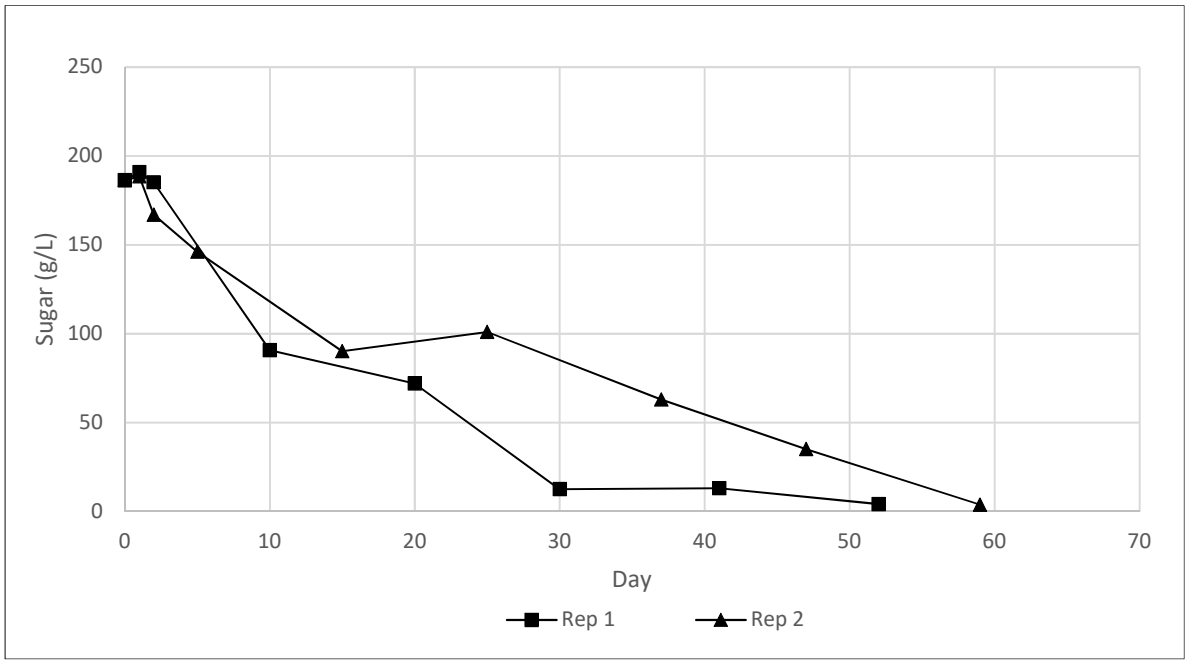

Figure 6. Sugar consumption of SJGM[1] fermentation replicates with no exogenous nitrogen added (control), fermented with strain W15, at 23°C.
[1]Synthetic grape juice media.
Rep 1 = replicate 1, rep 2 = replicate 2.

Effects of Nutrient Source, Temperature and Yeast Strain on YAN Consumption

Temperature was the only factor which YAN consumption rate and pattern corresponded to. For 18°C and 23°C treatments, the initial nitrogen content in SGJM and the first half of the addition were all consumed within 48 hours since inoculation. The second half of the addition, another 50 mg N/L of either PAN or AMM, were consumed within 48 hours of addition for the 18°C treatments, and within 24 hours of addition for the 23°C treatments. The day of addition varied between samples. Controls which had only the initial 50 mg N/L of PAN depleted YAN content within 24 hours of inoculation. The 12°C treatments had more variation in terms of YAN consumption rate, however no trend were observed. The first half of the addition was depleted 2 to 4 days after inoculation, and the second half of the addition was depleted within 2 days of addition. The presence of YAN after the second half addition was not detected in some samples, likely due to rapid consumption by yeast.

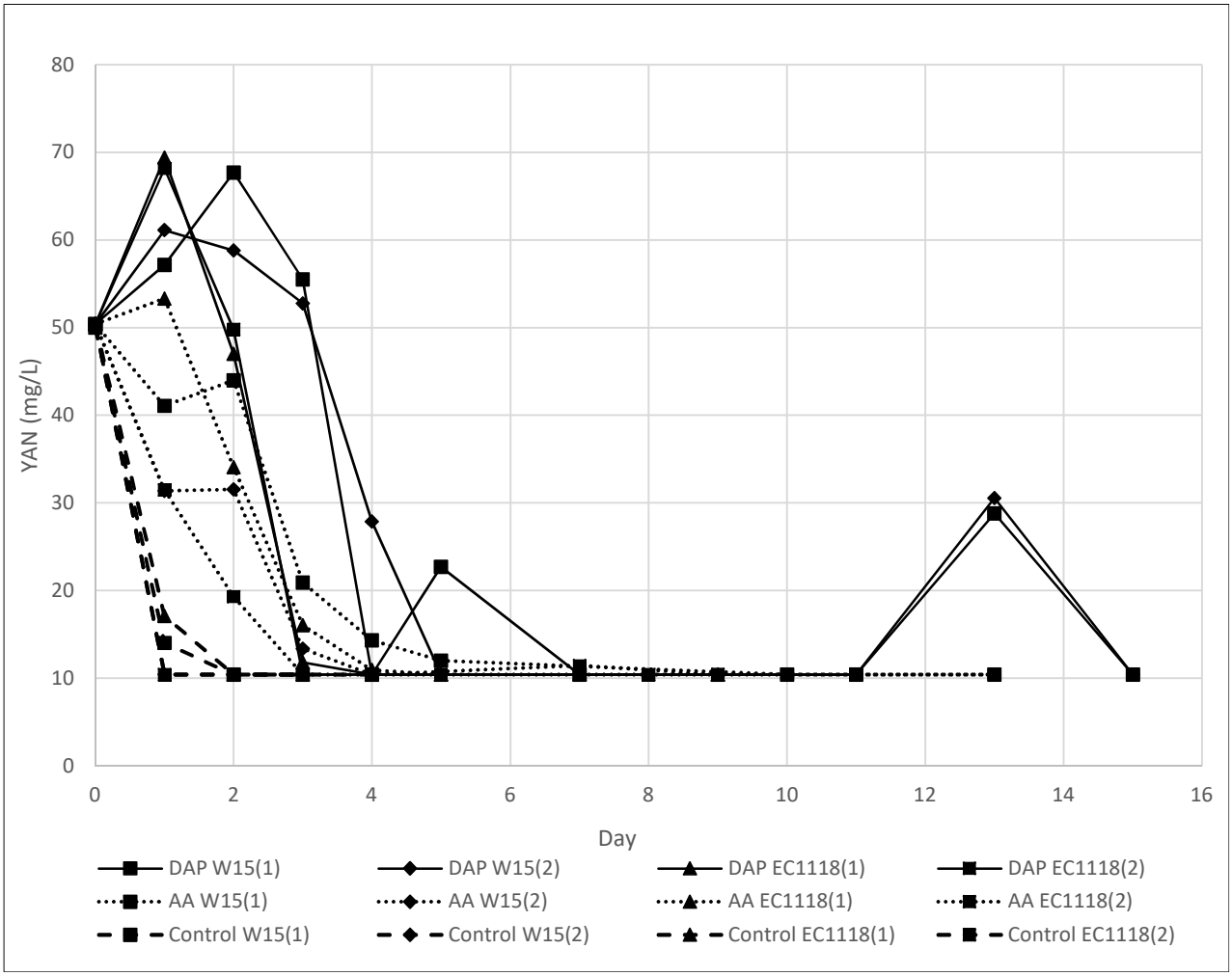

Figure 7. YAN consumption during fermentation of SGJM[1] by yeast strains EC1118 and W15, underwent three different nutrient treatments (DAP, Amino Acid mix) at temperature 12°C each treatment carried out with 2 replicates.

[1]Synthetic grape juice media.

AA = amino acid mix, DAP = diammonium phosphate, (1) = replicate 1, (2) = replicate 2.

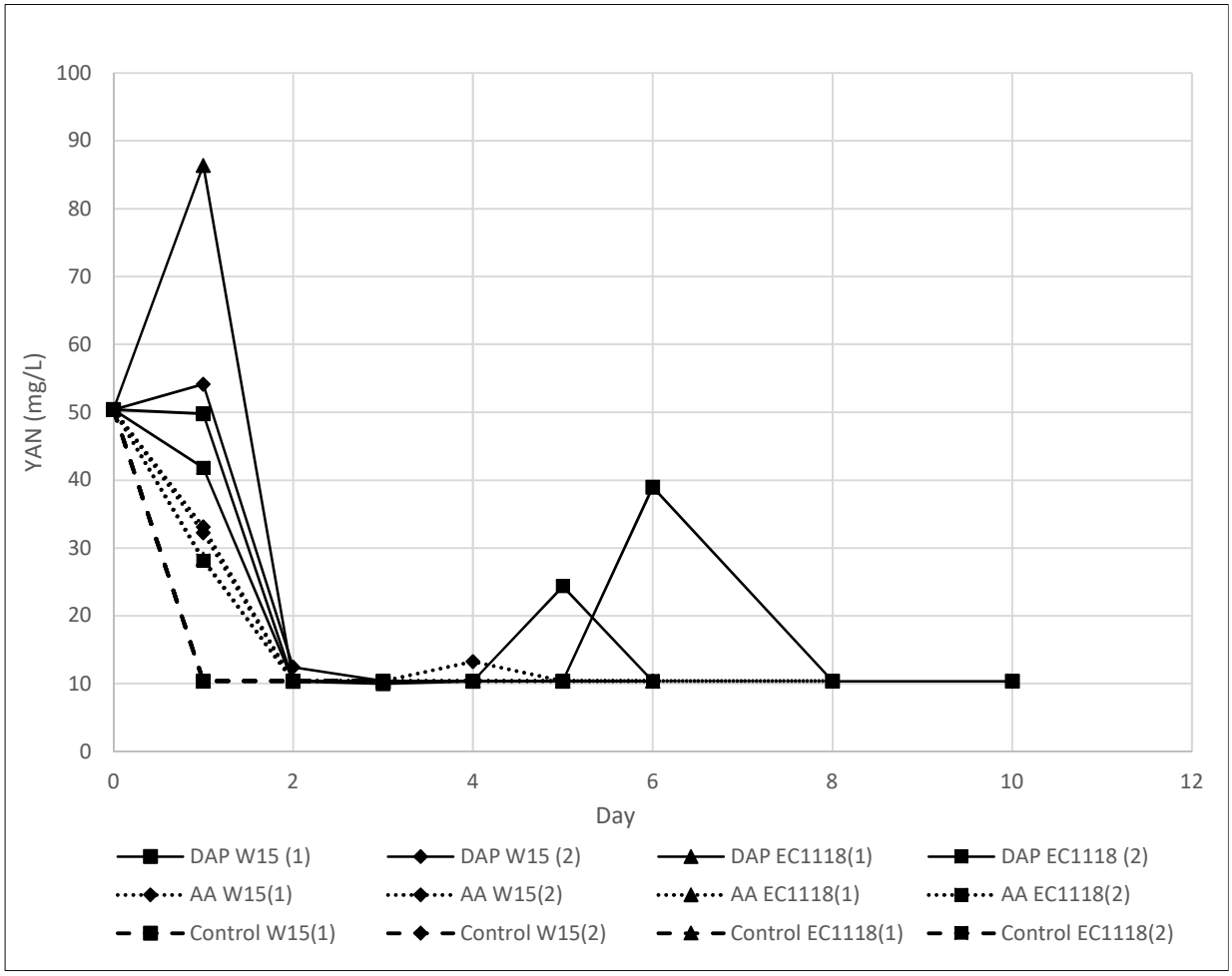

Figure 8. YAN consumption during fermentation of SGJM[1] by yeast strains EC1118 and W15, underwent three different nutrient treatments (DAP, Amino Acid mix) at temperature 18°C, each treatment carried out with 2 replicates.

[1]Synthetic grape juice media.

AA = amino acid mix, DAP = diammonium phosphate, (1) = replicate 1, (2) = replicate 2.

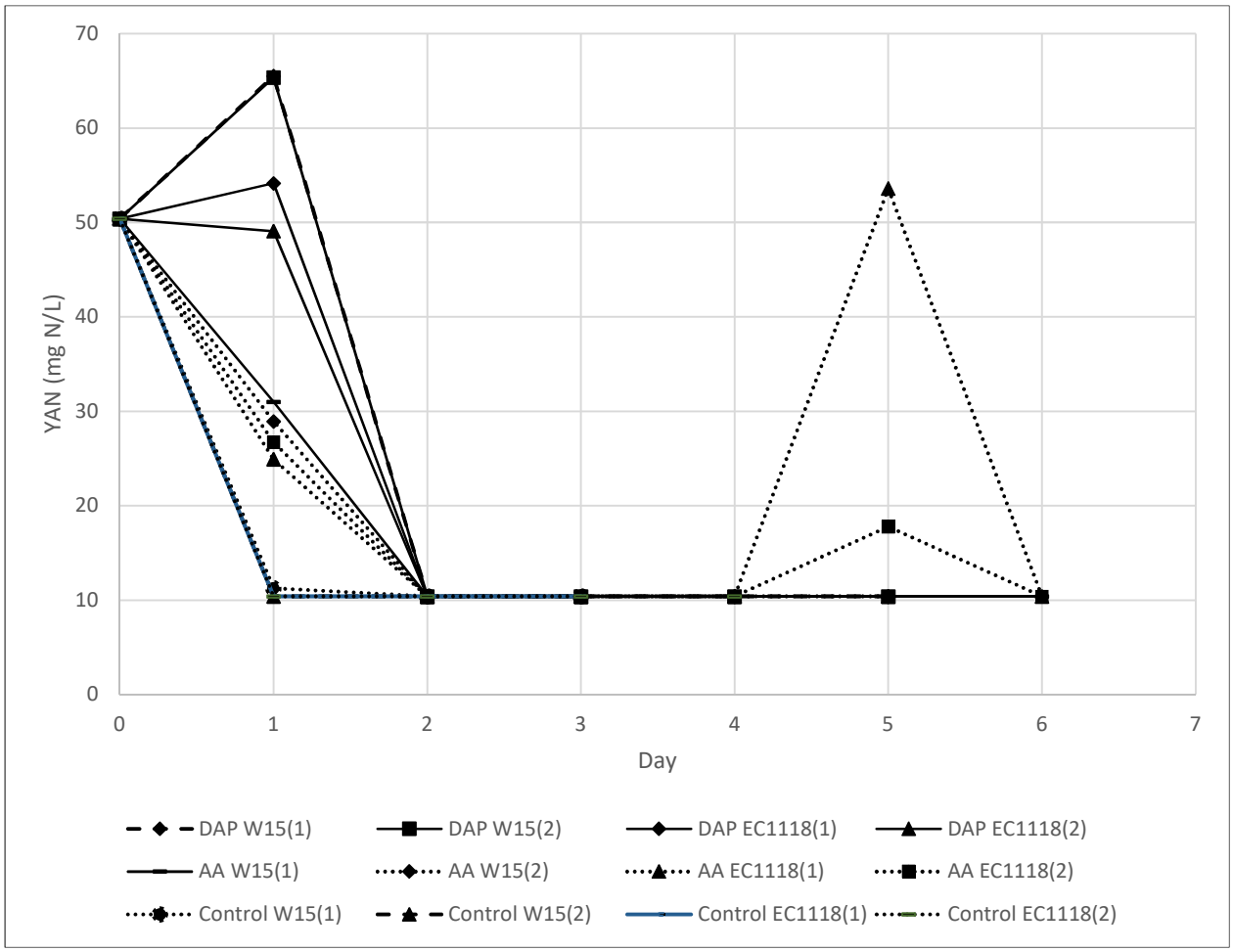

Figure 9. YAN consumption during fermentation of SGJM[1] by yeast strains EC1118 and W15, underwent three different nutrient treatments (DAP, Amino Acid mix) at temperature 23°C, each treatment carried out with 2 replicates.

[1]Synthetic grape juice media.

AA = amino acid mix, DAP = diammonium phosphate, (1) = replicate 1, (2) = replicate 2.

Effects of Nutrient Sources, Temperature and Yeast Strain on Final Wine Chemistry.

Acetic Acid. Although the p-value for all factors were less than 0.05, pairwise comparison of mean in acetic acid concentrations (g/L) showed that for both yeast strains, the control fermentations at 18°C had significantly higher acetic acid concentrations than fermentations at other temperatures. Specifically, the W15 18°C Control had an extremely high concentration, with a mean of 11.18 and standard error of 0.36, significantly higher than the other two nutrient treatments at the same temperature, and with the same yeast strain W15 18°C Amino Acid (AA) and W15 18°C DAP. Its concentration was also significantly higher than the EC1118 18°C Control treatment, with a mean difference of 9.64 and standard error of 0.51. The average acetic acid concentration was 0.472 g/L with a standard error of 0.0683 g/L (W15 18°C Control was excluded).

Table 1. Three-way ANOVA analysis of mean[1] in acetic acid concentration (g/L) of all treatments

FACTOR	DF	SUM SQ	MEAN SQ	F VALUE	PROBABILITY
NUTRIENT	2	24.756	12.3872	47.731	6.364e-08
YEAST	1	12.857	12.8574	49.579	1.436e-06
TEMPERATURE	2	33.735	16.8674	65.042	5.793e-09
NUTRIENT: YEAST	2	19.397	9.6893	37.398	3.888e-07
NUTRIENT: TEMPERATURE	4	68.01	17.0024	65.563	1.753e-10
YEAST: TEMPERATURE	2	21.285	10.6424	41.039	1.970e-07
NUTRIENT: YEAST: TEMPERATURE	4	39.666	9.9164	38.239	1.440e-08
RESIDUALS	18				

[1]mean average of duplicate fermentations analyzed by R package emmeans.

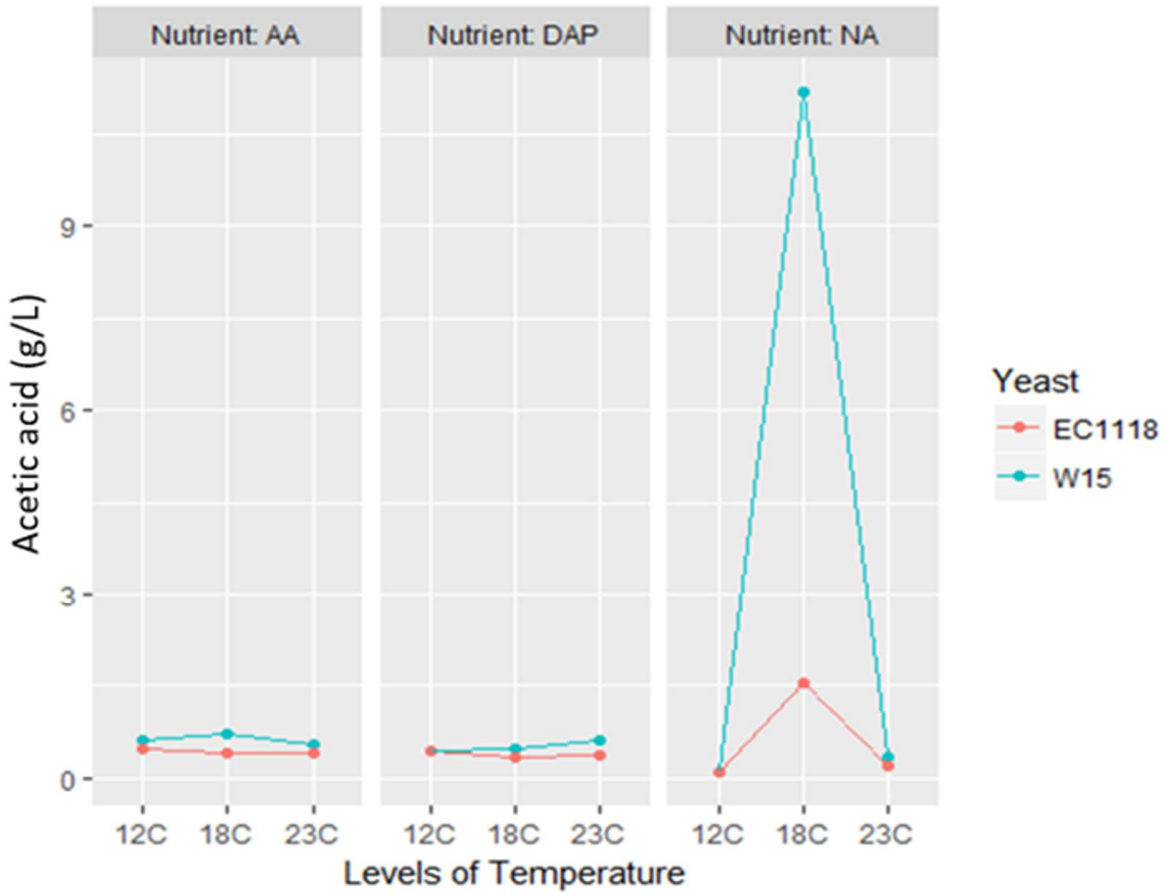

Figure 10. Mean in acetic acid concentration (g/L) of all treatments

Citric Acid. Yeast strain and interactive effects accounted for much of the variance in mean citric acid concentrations among treatments. Pairwise comparison demonstrated that only the 18°C treatments had pairs with significantly different mean citric acid concentrations, for yeast W15, AA treatment produced higher citric acid than control; for nutrient source AA, W15 produced higher citric acid than EC1118; for the control EC1118 produced higher citric acid than the W15 control. It is concluded that variation in citric acid concentration was greater at moderate fermentation temperature (18°C), however no specific trend was found. The average citric acid concentration was 0.16363 g/L, with a standard error of 0.00252 g/L.

Table 2. Three-way ANOVA analysis of mean[1] in citric acid concentration (g/L) of all treatments

FACTOR	DF	SUM SQ	MEAN SQ	F VALUE	PROBABILITY
NUTRIENT	2	0.00014607	0.00007304	0.6355	0.541136
YEAST	2	0.00123625	0.00061813	5.3786	0.014748
TEMPERATURE	1	0.00001431	0.00001431	0.1245	0.728262
NUTRIENT: YEAST	4	0.00078747	0.00019687	1.7140	0.190959
NUTRIENT: TEMPERATURE	2	0.00172156	0.00086078	7.4900	0.004297
YEAST: TEMPERATURE	2	0.00006502	0.00003251	0.2829	0.756896
NUTRIENT: YEAST: TEMPERATURE	4	0.00152461	0.00038115	3.3166	0.033473
RESIDUALS	18	0.00206862	0.00011492		

[1]mean average of duplicate fermentations analyzed by R package emmeans.

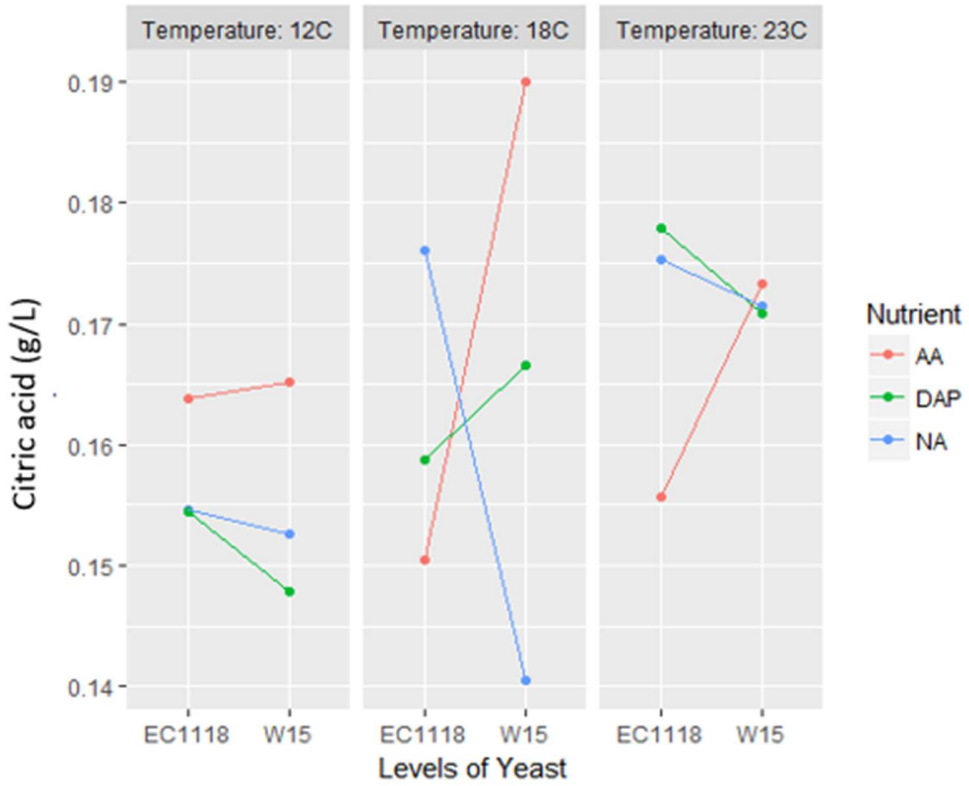

Figure 11. Mean in citric acid concentration (g/L) for all treatments

Ethanol. The controls at 12°C for both yeast strains and the control for W15 at 18°C had significantly lower ethanol content than other treatments, as they did not ferment to dryness. For the W15 control, there was a positive linear relationship between temperature and ethanol content. All supplemented treatments fermented to dryness regardless of yeast strain and fermentation temperature. As the initial sugar concentration was standardized to 180 g/L, the ethanol content after conversion remained largely constant across different treatments. Barring those that did not ferment to dryness, the mean in ethanol content was 9.55% with a standard error of 0.106%.

Table 3. Three-way ANOVA analysis of mean[1] in ethanol content (%) of all treatments

	DF	SUM SQ	MEAN SQ	F VALUE	PROBABILITY
NUTRIENT	2	75.956	37.978	112.8373	6.549e-11
YEAST	1	3.773	3.773	11.2109	0.0035786
TEMPERATURE	2	15.225	7.613	22.6178	1.227e-05
NUTRIENT: YEAST	2	10.553	5.277	15.6775	0.0001141
NUTRIENT: TEMPERATURE	4	43.602	10.901	32.3870	5.328e-08
YEAST: TEMPERATURE	2	5.469	2.735	8.1250	0.0030586
NUTRIENT: YEAST: TEMPERATURE	4	9.384	2.346	6.9701	0.0014245
RESIDUALS	18	6.058	0.337		

[1]mean average of duplicate fermentations analyzed by R package emmeans.

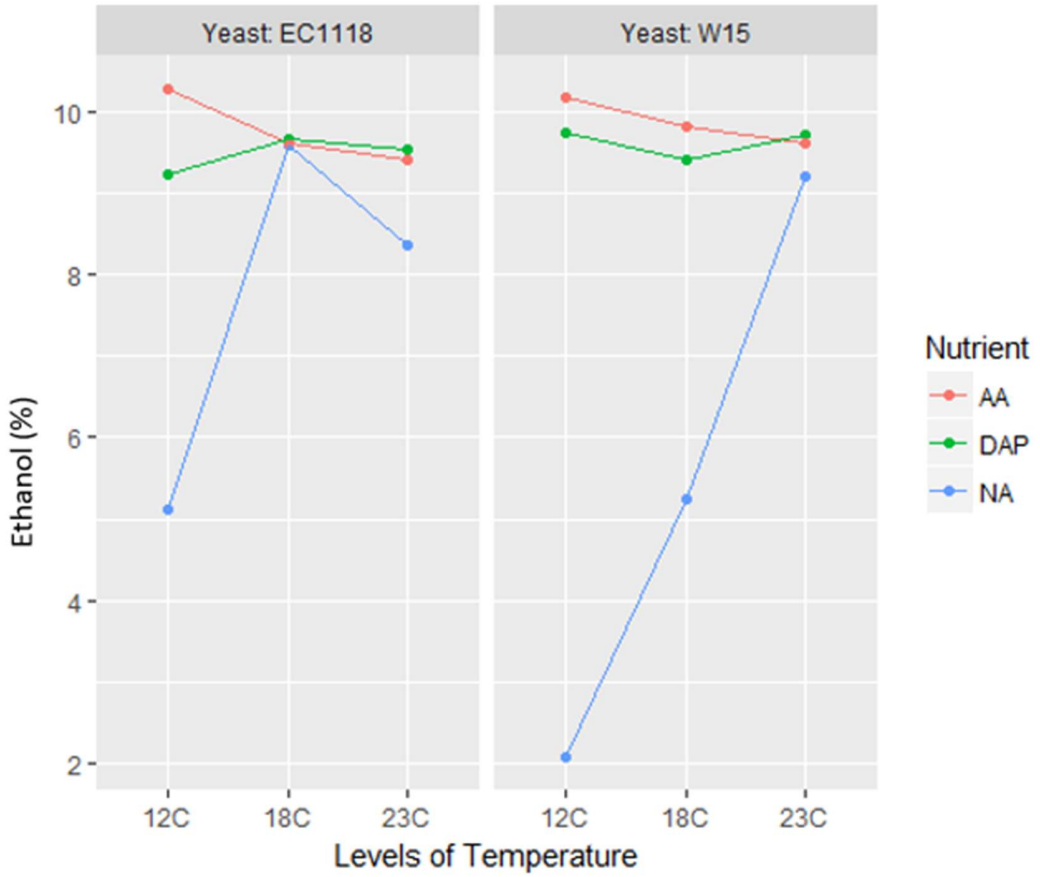

Figure 12. Mean in ethanol content (%) for all treatments

Lactic Acid. Yeast strain and fermentation temperature had the most impact of mean lactic acid concentration. For controls of both yeast strains, the 23°C treatment had a lactic acid concentration that significantly higher than the 12°C treatment. Treatments with exogenous nitrogen supplemented did not show any linear correlation. The average lactic acid concentration was 0.22572 g/L, with a standard error of 0.0236 g/L.

Table 4. Three-way ANOVA analysis of mean[1] in lactic acid concentration (g/L) of all treatments

	DF	SUM SQ	MEAN SQ	F VALUE	PR(>F)
NUTRIENT	2	0.022552	0.011276	1.1013	0.3538305
YEAST	1	0.035335	0.035335	3.4510	0.0796488
TEMPERATURE	2	0.274224	0.137112	13.3912	0.0002738
NUTRIENT: YEAST	2	0.001182	0.000591	0.0577	0.9440886
NUTRIENT: TEMPERATURE	4	0.087709	0.021927	2.1415	0.1174297
YEAST: TEMPERATURE	2	0.049555	0.024778	2.4199	0.1172766
NUTRIENT: YEAST: TEMPERATURE	4	0.007665	0.001916	0.1871	0.9420368
RESIDUALS	18	0.184301	0.010239		

[1]mean average of duplicate fermentations analyzed by R package emmeans.

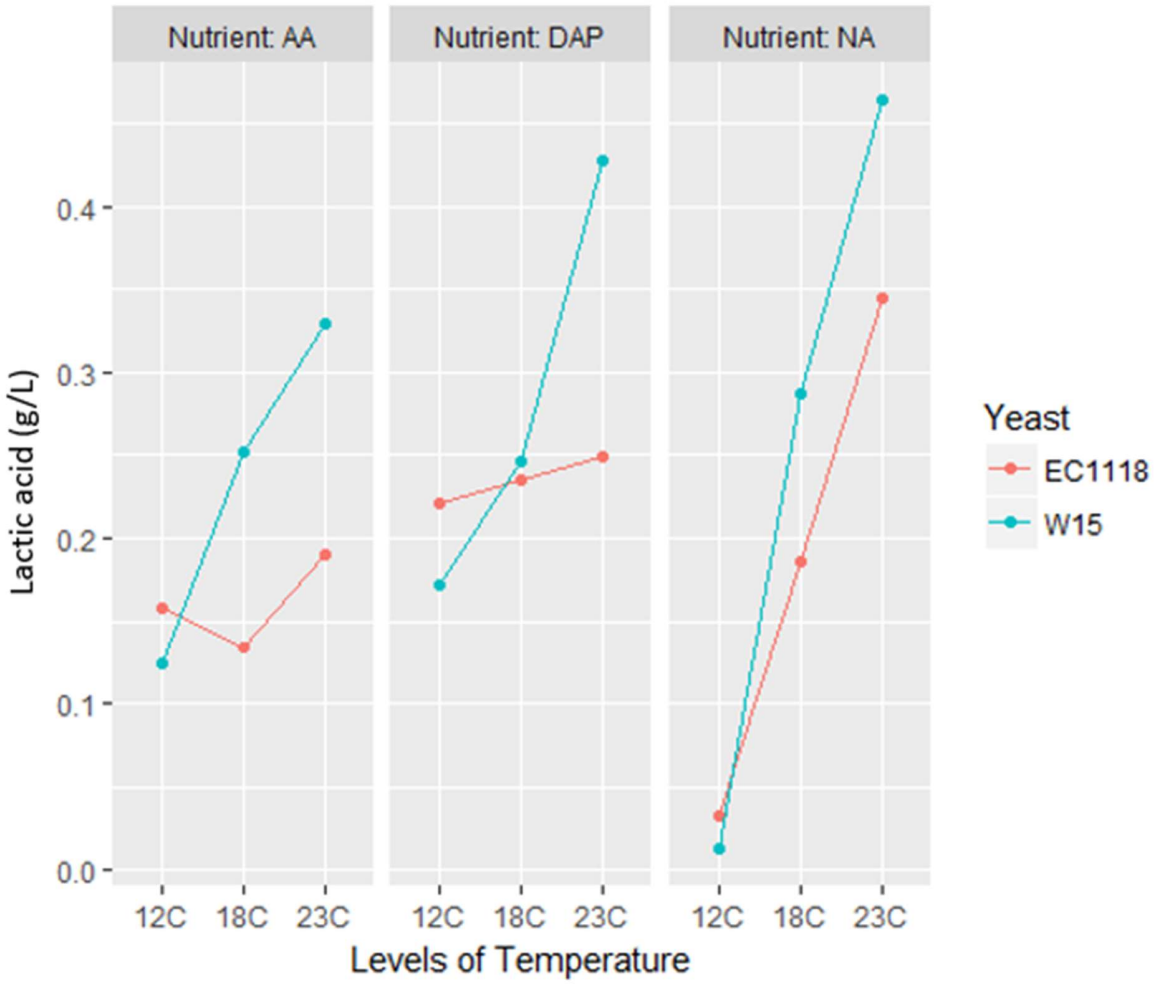

Figure 13. Mean in lactic acid concentration (g/L) for all treatments

Malic Acid. For all 18°C and 23°C treatments, W15 produced significantly higher malic acid concentrations than EC1118, regardless of nutrient type. The control fermentations for both yeast strains had higher malic acid at 23°C and 18°C than at 12°C. Among the W15 AA treatments, 18°C had higher malic acid concentration than 12°C and 23°C. Controls had the lowest malic acid concentration for all EC1118 18°C and 23°C treatments. Other significant differences between samples were observed, but no specific trend was found. Average malic acid concentration across all treatments was 2.53 g/L, with a standard error of 0.07 g/L.

Table 5. Three-way ANOVA analysis of mean[1] in malic acid concentration (g/L) of all treatments

	DF	SUM SQ	MEAN SQ	F VALUE	PR(>F)
NUTRIENT	2	0.63670	0.31835	26.7216	4.091e-06
YEAST	1	1.69525	1.69525	142.2963	5.554e-10
TEMPERATURE	2	0.93372	0.46686	39.1876	2.765e-07
NUTRIENT: YEAST	2	0.16411	0.08205	6.8874	0.006008
NUTRIENT: TEMPERATURE	4	0.85766	0.21442	17.9976	4.202e-06
YEAST: TEMPERATURE	2	0.69296	0.34648	29.0830	2.299e-06
NUTRIENT: YEAST: TEMPERATURE	4	0.20373	0.05093	4.2753	0.013205
RESIDUALS	18	0.21444	0.01191		

[1]mean average of duplicate fermentations analyzed by R package emmeans.

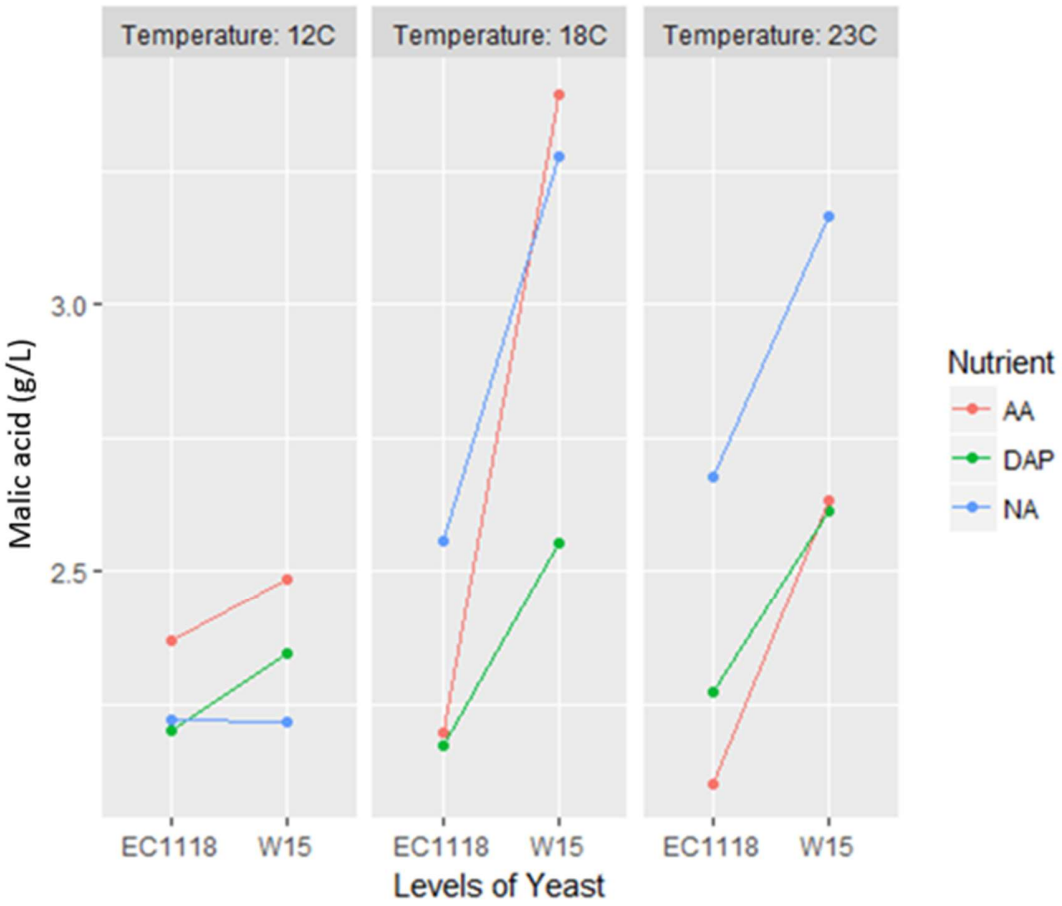

Figure 14. Mean in malic acid concentration (g/L) for all treatments

pH. While significant pH differences were observed, no trends were observed across fermentations. Most treatments fell within the range of 3.1 to 3.3, except one outlier, W15 18°C Control, with the pH of 2.875. The average pH was 3.16 with a standard error of 0.017.

Table 6. Three-way ANOVA analysis of mean[1] in pH of all treatments

	DF	SUM SQ	MEAN SQ	F VALUE	PR(>F)
NUTRIENT	2	0.078272	0.039136	16.1017	9.792e-05
TEMPERATURE	2	0.015239	0.007619	3.1349	0.0679001
YEAST	1	0.049136	0.049136	20.2160	0.0002793
NUTRIENT: TEMPERATURE	4	0.048411	0.012103	4.9794	0.0070121
NUTRIENT: YEAST	2	0.020039	0.010019	4.1223	0.0335813
TEMPERATURE: YEAST	2	0.010506	0.005253	2.1611	0.1441467
NUTRIENT: TEMPERATURE: YEAST	4	0.093744	0.023436	9.6423	0.0002386
RESIDUALS	18	0.043750	0.002431		

[1]mean average of duplicate fermentations analyzed by R package emmeans.

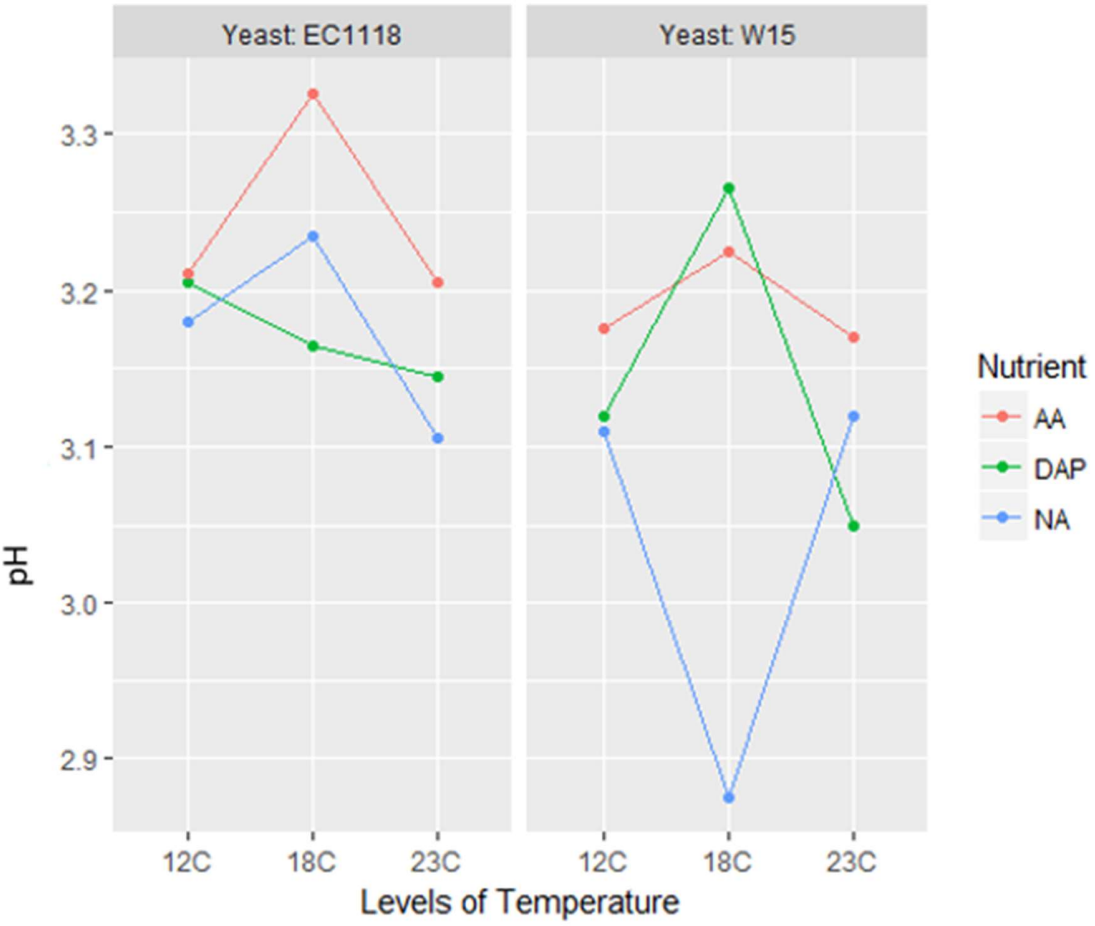

Figure 15. Mean pH for all treatments

Residual Sugar. The three control fermentations (W15 12°C, EC1118 12°C, and W15 18°C) that did not ferment to dryness had significantly higher residual sugar than the rest of the treatments. Mean in residual sugar for W15 12°C Control was 136.5 g/L, for EC1118 12°C Control was 80.5 g/L, and for W15 18°C Control was 52.5 g/L. Among treatments fermented to dryness, the average residual sugar concentration was 2.41 g/L with a standard error of 0.23 g/L.

Table 7. Three-way ANOVA analysis of mean[1] in residual sugar (g/L) of all treatments

	DF	SUM SQ	MEAN SQ	F VALUE	PROBABILITY
NUTRIENT	2	15801.8	7900.9	1703.933	< 2.2e-16
YEAST	1	1286.6	1286.6	277.479	2.204e-12
TEMPERATURE	2	7692.5	3846.3	829.495	< 2.2e-16
NUTRIENT: YEAST	2	2521.0	1260.5	271.843	3.565e-14
NUTRIENT: TEMPERATURE	4	16549.1	4137.3	892.255	< 2.2e-16
YEAST: TEMPERATURE	2	607.1	303.5	65.463	5.504e-09
NUTRIENT: YEAST: TEMPERATURE	4	1217.3	304.3	65.632	1.737e-10
RESIDUALS	18	83.5	4.6		

[1]mean average of duplicate fermentations analyzed by R package emmeans.

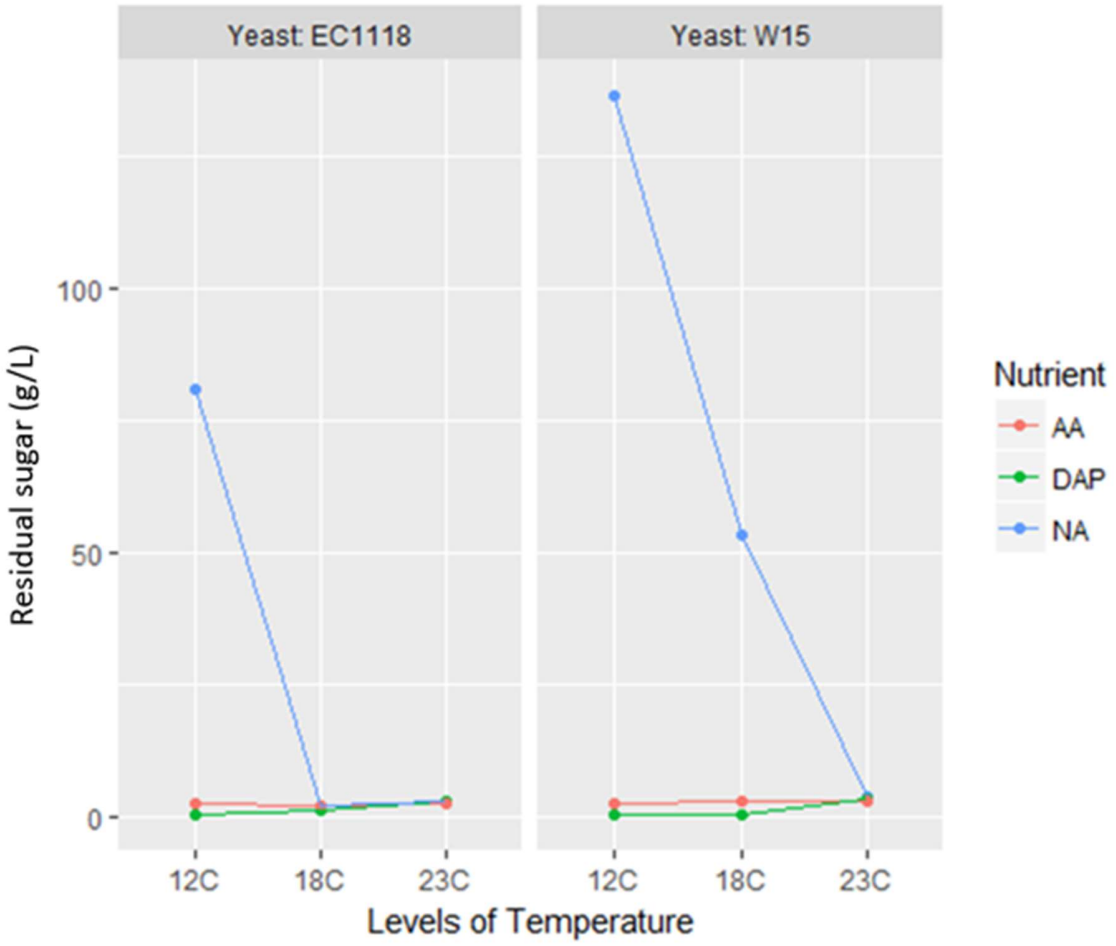

Figure 16. Mean in residual sugar (g/L) for all treatments

Titratable Acidity. Control treatments had higher titratable acidity than AA treatments in three instances: EC1118 at 18°C, W15 at 23°C, and W15 at 18°C. W15 fermentations had higher titratable acidity than EC1118 with AA at 18°C and controls at 18°C and 23°C. W15 18°C Control treatment produced a mean in titratable acidity of 23.55 g/L, which was significantly higher than all of the other treatments. With the exclusion of the W15 18°C control, average titratable acidity was 6.81 g/L with a standard error of 0.199 g/L.

Table 8. Three-way ANOVA analysis of mean[1] in titratable acidity (g/L) of all treatments

	DF	SUM SQ	MEAN SQ	F VALUE	PROBABILITY
NUTRIENT	2	95.203	47.601	76.065	1.659e-09
TEMPERATURE	2	97.848	48.924	78.189	1.331e-09
YEAST	1	63.441	63.441	101.39	8.030e-09
NUTRIENT: TEMPERATURE	4	129.782	32.446	51.854	1.225e-09
NUTRIENT: YEAST	2	47.513	23.756	37.967	3.484e-07
TEMPERATURE: YEAST	2	57.773	28.886	46.165	8.188e-08
NUTRIENT: TEMPERATURE: YEAST	4	80.800	20.2	32.283	5.463e-08
RESIDUALS	18	11.263	0.626		

[1]mean average of duplicate fermentations analyzed by R package emmeans.

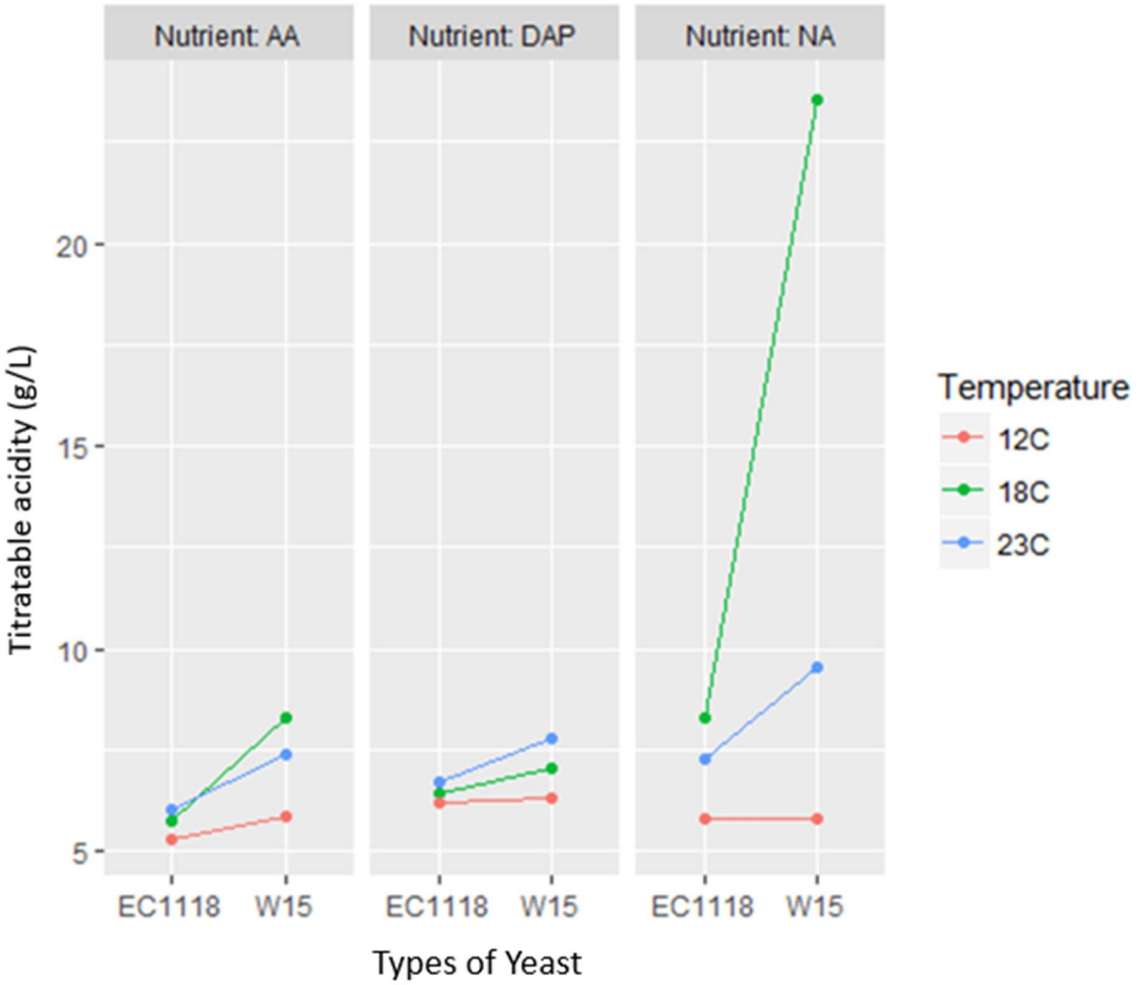

Figure 17. Mean in titratable acidity (g/L) for all treatments

DISCUSSSION

Sugar and YAN Consumption

Overall, yeast strain had a greater impact on the rate and pattern of sugar depletion than nutrient source. EC1118 was a more robust fermenter than W15 regardless of temperature and nutrient source. Only slight difference was found between fermentations supplemented with different nutrient sources but inoculated with the same yeast strain. Organic nutrient seemed to consume sugar slightly faster than inorganic nitrogen, however the discrepancy was neglectable when taking into account of other treatments and factors. Previous studies have reported that supplementation with a mixture of amino acids will yield higher fermentation kinetics, as amino acids can be incorporated into the metabolic cycle of yeast more rapidly (Jiranek & Henschke, 1995), no significant difference was observed in this work. Previous studies have also suggested that ammonium is the preferred form of nitrogen in early fermentation for yeast biomass formation, and primary amino nitrogen is preferred later during cell maintenance (Waterhouse, Sacks & Jeffery, 2016). In this work, however, no difference was found on the sugar depletion rate between treatments supplemented with different nutrient source, neither at the beginning of fermentation nor following 1/3 sugar depletion.

Fermentation duration and length was found to be a function of fermentation temperature, and at lower temperatures, yeast stain and temperature had an interactive impact on the length of fermentation. The rate of YAN consumption was also directly linked to fermentation temperature, with YAN depletion slower at lower temperatures. At moderate and high temperatures, a majority of the fermentations showed greatly depleted YAN concentrations within 24 hours of addition, signifying rapid consumption regardless of nutrient type and yeast strain, thus raise the question that if it really matters to follow industrial and manufacturing protocols, for using a mixture

of organic and inorganic nitrogen source, and perform nutrient addition at separate timing.

Final Wine Chemistry

Among the chemical parameters measured, acetic acid concentration had a strong relation with other final wine chemistry such as titratable acidity, due to one treatment W15 18°C Control, which yielded over 10g/L of acetic acid in the final wine. As acetic acid contributed to overall titratable acidity, correspondingly the TA for W15 18°C Control was also significantly higher than the others and had a lower pH.

In this treatment, the acetic acid concentration was similar to the other treatments during the first half of the fermentation, then acetic acid concentration increased rapidly beginning at day 62 (residual sugar was above 150 g/L). The EC1118 18°C control also produced a high amount of acetic acid compared to other EC1118 treatments, though the mean for EC1118 was still 10 g/L lower than W15. This high concentration might be due to the yeast strain's stress response in a nitrogen deficit environment, as such a stressful environment may induce spikes in acetic acid (Vilanova et al., 2012). It is reported that excessive nitrogen (especially in the form of ammonium) can also induce such responses, looking at data from other treatments it is concluded that 150mg N/L was a sufficient but not excessive amount of nitrogen to supplement (Vilanova et al., 2007). Another plausible explanation can be that during a stuck fermentation with high gravity, high osmotic stress is induced which increase the production of glycerol through glycolysis; as the formation of glycerol is linked to an excess of NAD+ to NADH, redox balance will need to be reestablished, and under an anaerobic winemaking condition, this is done by the formation of acetic acid by oxidizing acetaldehyde (Waterhouse, Sacks & Jeffery, 2016). Since glycerol production and viability under different temperature varies largely by yeast strain (Vilanova et al., 2007), and W15 has been reported as a yeast strain that produces high glycerol content

during fermentation (reference 17), strain differences in acetic acid production was expected.

Previous studies have found (Redzepovic et al., 2003) differences in transcriptional regulation of the malic enzyme genes from different *Saccharomyces* strains, and that malate degradation varies up to 40% between strains (Saayman and Viljoen-Bloom, 2017). Malic acid also serves as a precursor for succinic acid production during fermentation via malic acid metabolism (Waterhouse, Sacks & Jeffery, 2016). EC1118 has been reported a as non-degrader of malic acid (Redzepovic et al., 2003), whereas W15 is known for high succinic acid production (reference 19). However the observed differences in malic acid concentration between W15 and EC1118 was the opposite that what would expected, as W15 actually had significantly higher malic acid concentration than EC1118 under all 18°C and 23°C treatments, and the reason for this is unclear. The differences in malic acid concentration are assumed to be correlated to differences in titratable acidity between EC1118 and W15 treatments, as some of the W15 treatments also had higher overall titratable acidity than EC1118. However the accuracy of titratable acidity was impacted by other factors, which will be discussed in the following section.

HPLC analysis of tartaric acid concentration was not reported here due to inconsistency throughout the course of fermentation. The concentration of tartaric acid is expected to remain roughly constant during fermentation, but variation as high as 1.5 g/L was found between samples taken on consecutive days of the same treatment. Prior to HPLC analysis, samples were frozen for a long period; when samples were thawed prior to analysis, potassium tartrate crystals were apparent, and appeared not to fully dissolve during heating prior to analysis. Model wine solutions lack protein and polysaccharides to help prevent the re-precipitation of KHT, resulting in high inconsistency in the results. Consequently, the presence of KHT also influenced the accuracy of titratable acidity analysis of the final model wine samples. For future HPLC

analysis, tartaric acid should be prepared separated by diluting the sample 1:4 into 0.1M HCL or citric acid solution to ensure full solubility.

A combination of low temperature and nutrient deficiency will stress yeast, likely causing fermentation activity to slow or cease before all sugar are consumed, and therefore resulting in high concentration of residual sugar as well as low level of ethanol content. This was evident in the control fermentations W15 18°C, W15 12°C, and EC1118 12°C.

As differences observed in post-fermentation chemical composition could not be explained by a single factor alone, the interactions between yeast strain, nutrient source, and fermentation temperature should be taken into consideration when modulating a specific chemical parameter during wine production.

CONCLUSION

The use of exogenous nitrogen supplementation can be essential on affecting the final wine's chemical composition as well as fermentation kinetics. The choice of nutrient package with yeast and temperature can impact important final wine chemistry such as titratable acidity, pH, ethanol and residual sugar. Different choice of yeast strain can affect fermentation kinetics, malic acid concentration and acetic acid production; lower temperature can reduce the rate of sugar depletion and increase the length of fermentation, so it is an important factor for smaller scale producers who have potential concerns of tank lock. Some factors are affected by a three-way interaction; therefore winemakers should take them all into consideration in commercial winemaking in order to finetune their wines toward desired chemical properties. 150 mg N/L was sufficient for a cool climate Riesling to reach dryness smoothly, and the nutrient is usually consumed within 1-2 days since addition. Temperature has an impact on the rate of sugar consumption, that under very low temperature (in the study, 12°C), it took longer time for the YAN to be depleted.

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